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Scientific Opinion on Flavouring Group Evaluation 213, Revision 2 (FGE.213Rev2): Consideration of genotoxic potential for α,β-unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)

Abstract

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) of the European Food Safety Authority (EFSA) was requested to evaluate the genotoxic potential of 26 flavouring substances from subgroup 2.7 of FGE.19 in Flavouring Group Evaluation (FGE) 213. In the first version of FGE.213 the Panel concluded, based on available genotoxicity data, that a concern regarding genotoxicity could be ruled out for 11 substances [FL-nos: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168], but for the remaining 15 substances in subgroup 2.7 further genotoxicity data were required. Based on new submitted genotoxicity data, the Panel concluded in FGE.213Rev1 that the concern regarding genotoxicity could be ruled out for 13 substances in subgroup 2.7 [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] but not for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525]. In FGE.213Rev2, new data on maltol were considered and the Panel concluded that for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525] in food the concern for genotoxicity could be ruled out. Moreover, the Panel reconsidered the available data on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127], based on new data on the structurally related substance pulegone, and concluded that additional genotoxicity data are needed to rule out the concern for genotoxicity of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127].

Keywords: FGE.213, α,β-unsaturated alicyclic ketones, flavouring substances, safety evaluation, subgroup 2.7, FGE.19

Requestor: European Commission

Question numbers: EFSA-Q-2015-00138, EFSA-Q-2015-00139

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Summary

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Scientific Panel was asked to evaluate flavouring substances using the procedure referred to in Commission Regulation EC No 1565/2000 (hereafter 'the Procedure').

The Flavouring Group Evaluation (FGE) 213 concerns 26 substances, corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are α,β-unsaturated alicyclic ketones [Flavour Information System (FL)-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-nos: 02.106, 09.305 and 09.525].

In the first version of FGE.213 the Panel concluded that the genotoxicity concern for ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-nos: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] could be ruled out and the 11 substances could accordingly be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application was required in addition to an *in vivo* comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] could not be evaluated through the Procedure. Accordingly, additional data on genotoxicity were required for representatives of these 13 substances.

The Flavour Industry informed that it no longer supports the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. In FGE.213Rev1, one additional substance has been included in subgroup 2.7, *tr*-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

In FGE.213Rev1, the Panel evaluated the new data submitted by the Flavour Industry in response to the data request presented in FGE.213. Based on these new data, the Panel concluded that the genotoxicity concern could be ruled out for the representative substances β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] and the nine substances that they represent [FL-nos: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305].

In the case of maltol, positive results were observed in an *in vitro* micronucleus assay in human peripheral blood lymphocytes and in an *in vivo* micronucleus assay in mouse bone marrow after intraperitoneal application. Maltol was also tested in rats (administered by gavage) in a combined bone marrow micronucleus assay and comet assay in liver. Both tests showed negative results, but no clinical signs and no bone marrow toxicity were observed. To investigate the systemic exposure, plasma bioanalysis was performed, but results were inconsistent. Owing to the intended use of maltol as a food-flavouring agent, the *in vivo* study performed with administration of maltol by gavage is considered more relevant than the study performed by intraperitoneal application. Therefore, the Panel concluded in Revision 1 of this FGE that for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525] the concern for genotoxicity could not be ruled out.

The Flavour Industry has submitted a new plasma bioanalysis for maltol, which is evaluated in the present revision of FGE.213 (FGE.213Rev2). The Panel considered this new plasma bioanalysis and concluded that it seems justifiable to assume that animals were systemically exposed to maltol and that the bone marrow was exposed in the *in vivo* micronucleus assay. Therefore, the negative result of the *in vivo* micronucleus assay can be considered reliable and, accordingly, the concern for genotoxicity for maltol [FL-no: 07.014] and for maltyl isobutyrate [FL-no: 09.525] in food is ruled out; both substances were evaluated by JECFA before 2000 and no EFSA consideration is required.

Moreover, the Panel reconsidered the available data on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127], based on new data on the structurally related substance pulegone and concluded that additional genotoxicity data are needed to rule out the concern for genotoxicity on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127].

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The use of flavourings is regulated under Regulation (EC) No 1334/2008[1] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[2]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) N° 1565/2000.

On 10 April 2014 the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids adopted an opinion on Flavouring Group Evaluation 213, Revision 1 (FGE.213Rev1): Consideration of genotoxic potential for α,β-unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19.[3]

The Panel concluded that, for maltol [FL-no: 07.014] and for maltyl isobutyrate [FL-no: 09.525] the Panel's concern with respect to genotoxicity could not be ruled out and subsequently additional data are requested. In particular it was pointed out that the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

On 6 January 2015 the applicant has submitted additional data on the representative substance maltol [FL-no: 07.014] in response to this EFSA evaluation. This additional data regards a study intended to look at systemic exposure of rats following oral administration of this substance, using the same dosing regimen employed in the combined micronucleus and comet test previously submitted.

Terms of Reference as provided by the European Commission

The European Commission requests the European Food Safety Authority (EFSA) to evaluate this new information and, depending on the outcome, proceed to the full evaluation on this flavouring substance in accordance with Commission Regulation (EC) N° 1565/2000[4].

[1] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34-50

[2] EC (European Commission), 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1-161

[3] EFSA Journal 2014;12(2):3587.

[4] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8-16

2. Data and Methodologies

2.1. History of the evaluation of FGE.19 substances

Flavouring Group Evaluation (FGE) 19 (FGE.19) contains 360 flavouring substances from the European Union (EU) Register being α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008a).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The α,β-unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure–activity relationship ((Q)SAR) prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these α,β-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a, b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008b) could not be evaluated through the Procedure because of concerns with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data from the Flavour Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220 the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related α,β-unsaturated substances in the different subgroups for which additional data are requested, EFSA worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise, an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavour Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The Flavour Industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity.

2.2. History of the evaluation of the substances belonging to FGE.213

In the EFSA Opinion 'List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing' (EFSA, 2008c), representative flavouring substances have been selected for FGE.19 subgroup 2.7, corresponding to FGE.213.

In the first scientific opinion on FGE.213 (EFSA, 2009), the Panel concluded that, based on the data available, the concern with respect to genotoxicity could be ruled out for 11 substances [FL-nos: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 7.117, 07.118, 07.119, 07.120 and 07.168]. Nine of these substances have been evaluated by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) before 2000 to be of no safety concern and, in accordance with Commission Regulation (EC) No 1565/2000, no further consideration is requested. The remaining two substances, ethyl maltol [FL-no: 07.047] and 2-hydroxypiperitone [FL-no: 07.168], were evaluated in FGE.83Rev1 (EFSA CEF Panel, 2010) and FGE.11Rev2 (EFSA CEF Panel, 2011), respectively, using the Procedure.

For maltol [FL-no: 07.014], the Panel requested a combined *in vivo* micronucleus and comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

For the remaining 13 substances [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] additional data on genotoxicity were required for the representative substances, according to the Opinion of the CEF Panel on the 'Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19' (EFSA, 2008b).

FGE	Adopted by EFSA	Link	No. of Substances
FGE.213	27 November 2008	http://www.efsa.europa.eu/en/efsajournal/pub/879.htm	26
FGE.213Rev1	10 April 2014	http://www.efsa.europa.eu/it/efsajournal/pub/3661.htm	26
FGE.213Rev2	09 September 2015	http://www.efsa.europa.eu/it/efsajournal/pub/4244.htm	26

In FGE.213 Revision 1 (FGE.213Rev1) the Panel evaluated additional genotoxicity data submitted by the Flavouring Industry (IOFI, 2012, 2013) in response to a data request presented in FGE.213 (EFSA, 2009).

The new data submitted concerned five of the original six representative substances requested by the Panel (EFSA, 2008c), namely β-ionone [FL-no: 07.008], maltol [FL-no: 07.014], β-damascone [07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] (Table 1).

The Flavour Industry informed that it no longer supports the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. However, since piperitenone oxide was a self-representative substance, this did not affect the evaluation of the remaining substances in FGE.213Rev1.

In FGE.213Rev1, one additional substance was included in subgroup 2.7, tr-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

In FGE.213Rev1, the Panel concluded that the *in vitro* and *in vivo* genotoxicity data for the selected representative substances β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] do not indicate a genotoxic potential. Therefore, these substances, and the nine substances that they represent [FL-no: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305] could be evaluated through the Procedure.

During the evaluation of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127], using the Procedure (in FGE.57Rev1), the Panel noted that the chemical structure of *p*-mentha-1,4(8)-dien-3-one [FL-no:

07.127] is actually more closely related to the structure of pulegone (Table 2) than to the structures used for the read-across approach in FGE.213Rev1. New information (NTP, 2011) was found on genotoxicity and carcinogenicity of pulegone, from which additional data are expected to be provided by the applicant. The data available, at present, on pulegone and on the structurally related *p*-mentha-1,4(8)-dien-3-one [FL-no 07.127] do not rule out the concern for genotoxicity and carcinogenicity. Therefore, the genotoxicity of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] will be reconsidered based on additional data.

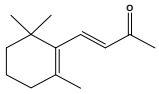
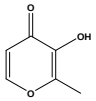
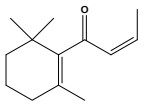
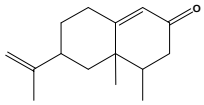
For maltol [FL-no: 07.014], a combined bone marrow micronucleus test and comet assay in rats (Beevers, 2013a) was evaluated by the Panel in FGE.213Rev1. The results of the micronucleus test showed that there were no statistically significant increases in micronucleus frequency for any dose group after oral treatment with maltol when compared with the vehicle control group. The comet assay did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis, and the groups treated with maltol showed mean percentage tail intensities and tail moments that were similar to vehicle controls and fell within historical control ranges.

The Panel noted that, at the dose levels selected, no clinical signs and no bone marrow toxicity were observed in any animal in the maltol-treated groups, which may reflect the possibility that the bone marrow and the liver were not exposed. Therefore, plasma analysis for proof of exposure was requested. Analysis of maltol in plasma was performed using a gas chromatography with mass selective detection (GC–MSD) method. Results showed marked inconsistencies among animals and between sampling times. The Panel concluded that negative findings observed in the combined bone marrow micronucleus test and comet assay in the liver of treated rats could not rule out the concern for genotoxicity of maltol since the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

Following the Panel's conclusion in FGE.213Rev1, the Flavour Industry has submitted a new plasma analysis (Beevers, 2015) performed on the same strain of rats and using the same dosing regimen of the combined micronucleus test and comet assay (Beevers, 2013a). These new data are evaluated in this revision of FGE.213, FGE.213Rev2.

The new data provided show that detectable levels of maltol were found in all plasma samples isolated at 0.5, 1 and 2 hours after dosing. Peak plasma levels of maltol were seen in the majority of animals at 0.5 hours after dose administration. The concentration of maltol detected in plasma was different between the animals of 2 separate cages and the authors of the study did not identify any technical reasons that could account for this difference. The new data submitted are described and evaluated in Section 3 of the present revision. Sections 2.4 and 2.5 report the same information that was present in FGE.213 and FGE.213Rev1, respectively.

Table 1: Representative substances for subgroup 2.7 of FGE.19

FL-no JECFA-no	EU Register name	Structural formula	Comments
07.008 **389**	β-Ionone		*In vitro* assays in bacteria and mammalian cells submitted
07.014 **1480**	Maltol		*In vitro* assays in bacteria and mammalian cells and an *in vivo* combined comet and micronucleus assay submitted
07.083 **384**	β-Damascone		*In vitro* assays in bacteria and mammalian cells and an *in vivo* combined comet and micronucleus assay submitted
07.089 **1398**	Nootkatone		*In vitro* assays in bacteria and mammalian cells submitted

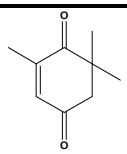
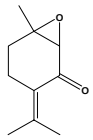
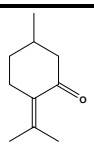
FL-no JECFA-no	EU Register name	Structural formula	Comments
07.109 1857	2,6,6-Trimethylcyclohex-2-en 1,4-dione		*In vitro* assays in bacteria and mammalian cells submitted
16.044 1574	Piperitenone oxide		No longer supported by the Flavour Industry and no data submitted

Table 2: Supporting substance for subgroup 2.7 of FGE.19

FL-no JECFA-no	Substance name	Structural formula	Comments
Not in Register 753	Pulegone		Additional *in vitro* and *in vivo* data (NTP, 2011)

2.3. Presentation of the substances in flavouring group evaluation 213

2.3.1. Description

The Flavouring Group Evaluation 213 (FGE.213) concerned 26 substances (Table 4), corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] (one substance [FL-no 16.044] is no longer supported by the Flavour Industry and one new substance [FL-no 07.224] has been included in Revision 1) and three are precursors for such ketones [FL-nos: 02.106, 09.305 and 09.525]. Two of these substances [FL-nos: 02.106 and 09.305] are precursors of the ketone β-ionone [FL-no: 07.008] and one [FL-no: 09.525] is a precursor of the ketone maltol [FL-no: 07.014]. Ten of the ketones have the possibility for keto–enol tautomerism [FL-nos: 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168]. Based on experimental evidence for other diketones it is anticipated that the enol is the predominant form.

Twenty-two of the substances in FGE.213 (including the new substance [FL-no 07.224], excluding [FL-no 16.044]) have formerly been evaluated by the JECFA (JECFA, 1999, 2001, 2006a, b, 2009a), a summary of their current evaluation status by the JECFA is given in Table 5.

As the α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a) the available data on genotoxic or carcinogenic activity for the 26 unsaturated alicyclic ketones and precursor in subgroup 2.7 will be considered in this FGE.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni and Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on 22 ketones [FL-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168 and 07.200]. The 22 ketones and their (Q)SAR predictions are shown in Table 6.

2.4. Data evaluated by the Panel in FGE.213[5]

2.4.1. (Q)SAR predictions

In Table 6 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS-Local Model–Ames test, DTU–NFI-MULTICASE–Ames test, Chromosomal aberration test (using Chinese hamster ovary (CHO) cells), Chromosomal aberration test (using Chinese hamster lung (CHL) cells) and Mouse lymphoma test) are presented.

Maltol [FL-no: 07.014], ethyl maltol [FL-no: 07.047] and nootkatone [FL-no: 07.089] were predicted positive with the MultiCASE model on chromosomal aberrations in CHL cells. All other predictions were negative or the substances were out of domain.

2.4.2. Genotoxicity studies

In subgroup 2.7 there are studies available for four substances. For maltol [FL-no: 07.014] eight *in vitro* and three *in vivo* studies have been evaluated. For ethyl maltol [FL-no: 07.047] two *in vitro* and one *in vivo* study were evaluated. Two *in vitro* studies concerning β-ionone [FL-no: 07.008] and one *in vitro* study for 3-methylcyclopentan-1,2-dione [FL-no: 07.056] were evaluated.

Study validation and results are presented in Tables 7 and 8.

In studies that were considered valid, the following results were obtained:

- Maltol-induced gene mutations in bacteria (Bjeldanes and Chew, 1979) and sister chromatid exchanges (SCEs) in human lymphocytes (Jansson et al., 1986). *In vivo*, maltol-induced micronuclei in mouse bone marrow after intraperitoneal application (Hayashi et al., 1988). Negative results were obtained in a sex-linked recessive lethal mutation assay in *Drosophila* (Mason et al., 1992). However, the micronucleus assay is considered more relevant than the *Drosophila* assay.
- Ethyl maltol-induced gene mutations in bacteria (Bjeldanes and Chew, 1979).
- A negative result was obtained with β-ionone in a gene mutation assay in bacteria (Mortelmans et al., 1986).

The validity of other studies was limited or could not be evaluated.

2.4.3. Carcinogenicity studies

In a combined study of developmental toxicity and carcinogenicity, three successive generations of male and female Charles River CD-COBS rats received 3-ethyl-2-hydroxy-2-cyclopenten-1-one (owing to keto–enol tautomerism this substance can exist as two isomers; the keto-isomer is 3-ethylcyclopentan-1,2-dione [FL-no: 07.057], a synonym for the keto-isomer is ethylcyclopentenolone) in the basal diet at doses of 0 (untreated control), 0 (propylene glycol control), 30, 80 or 200 mg/kg body weight (bw) per day. The F1 generation was initially exposed *in utero*, subsequently via the dams' milk until weaning, and then treated for two years and bred twice (at days 99 and 155). In the F1 generation, there were 100 animals of each sex in the untreated control group, 50 animals of each sex in both the propylene glycol control and 3-ethyl-2-hydroxy-2-cyclopenten-1-one-treated groups. Survival, clinical symptoms, food consumption, reproductive performance, haematological and clinical chemistry parameters were not adversely affected. Gross pathological and histopathological examination revealed no significant treatment-related effects. The incidence of benign or malignant tumours in treated animals was similar to that in controls. The No Observed Effect Level (NOEL) was 200 mg/kg bw per day (King et al., 1979).

The Panel concluded that 3-ethyl-2-hydroxy-2-cyclopenten-1-one (3-ethylcyclopentan-1,2-dione [FL-no: 07.057]) was not carcinogenic in rats under the study conditions.

Groups of 25 male and female rats were fed for two years on diets containing ethyl maltol [FL-no: 07.047] calculated to deliver 0, 50, 100 and 200 mg ethyl maltol/kg bw/day. No abnormalities were

[5] The data presented in Section 2.4 are cited from the first version of the present FGE.213. These data are the basis for the conclusions in FGE.213 requesting additional genotoxicity data.

seen as regards survival, clinical appearance, growth rate or food consumption, clinical chemistry, haematology and urinalysis. No histopathological changes and no increases in neoplasms were seen after the treatment with ethyl maltol (Gralla et al., 1969).

Study validation and results are presented in Table 9.

The Panel noted that this study was performed before Organisation for Economic Co-operation and Development (OECD) test guidelines 451/453 (1981a, b) had been established and it does not meet the criteria of these OECD test guidelines with respect to the number of animals. However, the Panel concluded that ethyl maltol was not carcinogenic in rats in this study.

2.4.4. Conclusion on genotoxicity and carcinogenicity

For the substances of this group, the applicability of the (Q)SAR models is very limited since many substances were out of domain in the ISS model and the MultiCASE models.

Two substances [FL-nos: 02.106 and 09.305] are precursors of β-ionone [FL-no: 07.008] and therefore, the conclusions for these two precursors could be based on the conclusions drawn for the corresponding ketone [FL-no: 07.008]. Maltyl isobutyrate [FL-no: 09.525] is a precursor of maltol [FL-no: 07.014], and accordingly, the conclusion for maltyl isobutyrate could be based on the conclusion drawn for maltol.

Maltol and ethyl maltol were considered separately because, in contrast to the other substances in this subgroup, they contain a ring-oxygen atom.

A carcinogenicity study on ethyl maltol [FL-no: 07.047] in rats (Gralla et al., 1969) has been evaluated. Although there were fewer animals per group than that suggested in OECD guidelines 451/453 (1981a, b), the study was in accordance with the standards available at that time. The Panel concluded that the result from this study could overrule the mutagenicity observed with ethyl maltol in bacteria, but not the mutagenicity observed with maltol [FL-no: 07.014] *in vitro* and *in vivo*. Since the micronuclei induced by maltol in mice were analysed after intraperitoneal application, a micronucleus assay after oral application is required, in addition to an *in vivo* comet assay, in order to clarify the genotoxic potential of maltol. A combination of the micronucleus assay and the comet assay in a single study would also be acceptable. The results of these assays would also be applicable to maltyl isobutyrate [FL-no: 09.525], which is a precursor of maltol.

No carcinogenicity was observed with 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] in rats. This substance was considered representative for nine substances [FL-nos: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080]. Therefore, the Panel concluded that the structural alert for genotoxicity is overruled for 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] as well as for the nine structurally related substances.

For the 13 remaining substances (including two precursors of a ketone) [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] a genotoxic potential could not be ruled out since only one valid negative bacterial genotoxicity study on [FL-no: 07.008] is available for these substances.

2.4.5. Conclusion based on the data available to the Panel in FGE.213

The Panel concluded that ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-nos: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] can be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application is required, in addition to an *in vivo* comet assay, in order to clarify the genotoxic potential. A combination of the micronucleus assay and the comet assay in a single study would also be acceptable. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

At present, the remaining 13 substances (including two precursors of a ketone) [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] cannot be evaluated using the Procedure. Additional data on genotoxicity are requested for the representative substances of this subgroup according to the opinion of the Panel on the 'Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19' (EFSA, 2008b).

2.5. Additional genotoxicity data evaluated by the Panel in FGE.213Rev1[6]

2.5.1. Presentation of the additional data

Based on Panel requirements published in FGE.213 (EFSA, 2009), additional data have been provided by the Flavour Industry for the representative substances: β-ionone [FL-no: 07.008], maltol [FL-no: 07.014], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109].

FGE.213, Revision 1 (FGE.213Rev1), includes the assessment of these additional genotoxicity studies (Table 3).

Table 3: Studies evaluated in FGE.213Rev1

Substance/study type	Bacterial Reverse Mutation assay	*In Vitro* Micronucleus test	*In Vivo* combined Micronucleus test and Comet assay
β-Ionone [FL-no: 07.008]	Ballantyne, 2011	Stone, 2011a	
Maltol [FL-no: 07.014]	Ballantyne, 2012	Whitwell, 2012	Beevers, 2013a
β-Damascone [FL-no: 07.083]	Bowen, 2011b	Stone, 2012	Beevers, 2013b, c
Nootkatone [FL-no: 07.089]	Marzin, 1998	Stone, 2011b	
2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]	Bowen, 2011a	Lloyd, 2011	

2.5.2. *In vitro* data

Bacterial reverse mutation assay

β-Ionone [FL-no: 07.008]

β-Ionone [FL-no: 07.008] was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2011). In the first experiment, the concentrations used were 0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate of β-ionone and the plate incorporation methodology was used. Toxicity ranging from slight thinning of the background lawn to complete killing of the tester strains was observed at 1 000 and/or 5 000 µg/plate for all tester strains in the absence and presence of S9-mix. In the second experiment, the concentrations tested were 10.24, 25.6, 64, 160, 400 and 1 000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method. Toxicity ranging from thinning of the background lawn and/or reduction in revertant numbers to complete killing of the tester bacteria occurred in all strains at 1 000 µg/plate in the absence and presence of S9-mix, and was also seen down to 160 and/or 400 µg/plate for some individual strains. The study design complied with current recommendations and an acceptable highest concentration was achieved. There was clearly no evidence of any mutagenic effect induced by β-ionone in any of the strains, either in the absence or presence of S9-mix.

Maltol [FL-no: 07.014]

Maltol [FL-no: 07.014] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2012). In the first experiment, the concentrations were 0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate of maltol and the plate incorporation methodology was used. Toxicity in the form of reduction of the number of revertants in the tester strain TA102 was observed at concentrations of 200 µg/plate and greater in the presence of

[6] The data presented in Section 2.5 are cited from FGE.213Rev1. These data are the basis for the conclusions in FGE.213Rev1 requesting additional data.

S9-mix and 1 000 and 5000 µg/plate in the absence of S9-mix. In the second experiment, the concentrations were 51.2, 128, 320, 800, 2 000 and 5 000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method in all strains. In tester strain TA102 an additional lower concentration of 20.48 µg/plate was incorporated into the testing protocol in both the absence and presence of S9-mix to assess, more carefully, the toxicity observed in experiment 1. Toxicity in the form of thinning of the background lawn and/or reduction in numbers of revertants occurred at 5 000 µg/plate in strain TA102 in the absence and presence of S9-mix, and in strain TA100 only in the presence of S9-mix. The study design complied with current recommendations and an acceptable highest concentration was achieved. There was no evidence of any mutagenic effect induced by maltol in any of the strains, either in the absence or presence of S9-mix.

β-Damascone [FL-no: 07.083]

An Ames assay was conducted in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA102 to assess the mutagenicity of β-damascone (purity: 95 %), both in the absence and in the presence of metabolic activation by S9-mix, in three separate experiments (Bowen, 2011b). The assay was performed according to OECD Guideline 471 (1997a) and according to Good Laboratory Practice (GLP) principles.

An initial experiment was carried out both in the absence and presence of S9-mix activation in all five strains, using final concentrations of β-damascone between 0.32 and 5000 µg/plate (0.32, 1.6, 8, 40, 200, 1 000, 5 000 µg/plate), plus negative (solvent) and positive controls. Evidence of toxicity was observed through thinning of the background lawn to complete killing at concentrations of 1 000 µg/plate and greater for strains TA1535, TA1537 and TA102 and/or 5000 µg/plate for strains TA98 and TA100 in the absence and presence of S9-mix. In the second experiment the highest concentration was retained for strains TA98 and TA100 in the absence and presence of S9-mix. In all other tester strains, the highest concentration was reduced to 2500 µg/plate based on toxicity observations. In addition, more narrow concentration intervals were used, starting at either 78.13 µg/plate or 156.3 µg/plate (78.13, 156.3, 312.5, 625, 1250, 2500 µg/plate). The standard plate incorporation assay was used in the first experiment but a pre-incubation step with S9-mix activation treatment was added in the second experiment to increase the chance of detecting a positive response. Evidence of toxicity was observed in TA98 at 625 µg/plate (in the presence of S9-mix) and at concentrations of 1250 µg/plate and greater (in the absence of S9-mix). Evidence of toxicity was observed in strains TA1535, TA1537 and TA102 at 625 µg/plate in the absence and presence of S9-mix. In strain TA100 toxicity was observed at concentrations of 1250 µg/plate and greater (in the presence of S9-mix) and at concentrations of 2500 µg/plate and greater (in the absence of S9-mix).

The third experiment was conducted using strain TA98 in the presence of S9-mix activation using the pre-incubation method. The maximum test concentration was reduced to 1 250 µg/plate based on toxicity observed in the previous experiments. In addition, more narrow concentration intervals were used, covering 19.53 to 1 250 µg β-damascone/plate (19.53, 39.06, 78.13, 156.3, 312.5, 625 and 1 250 µg/plate). Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. In all three experiments, no statistically significant increases in revertant numbers were observed at any concentration, in any of the strains, either in the presence or absence of S9-mix activation.

The Panel agreed with the conclusion of the study authors that β-damascone did not induce mutations in five strains of *S. typhimurium*, when tested under the conditions of this study.

Nootkatone [FL-no: 07.089]

Nootkatone [FL-no: 07.089] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence or presence of S9-mix (Marzin, 1998). A preliminary toxicity test to identify appropriate concentrations for the mutagenicity assays was performed in the absence and presence of S9-mix, and cytotoxicity was observed at 50 µg/plate in the absence of S9-mix and at 150 µg/plate in the presence of S9-mix. In the first mutagenicity experiment using plate incorporation methodology the concentrations tested were 0.5, 1.5, 5, 15 and 50 µg/plate in the absence of S9-mix metabolic activation and 1.5, 5, 15, 50 and 150 µg/plate in the presence of S9-mix. In the second experiment the plate incorporation method was used in the absence of S9-mix and the concentrations were 0.5, 1.5, 5, 15 and 50 µg/plate. While the pre-incubation method was used in the presence of S9-mix and

the concentrations were 0.5, 1.5, 5, 15, 50 and 150 µg/plate. Thus, the study design complied with current recommendations and an acceptable highest concentration was achieved. There was no evidence of any mutagenic effect induced by nootkatone in any of the strains, either in the absence or presence of S9-mix.

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Bowen, 2011a). In the first experiment, the concentrations tested were 0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate and plate incorporation methodology was used. In the second experiment, the concentrations were 156.3, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate of 2,6,6-trimethylcyclohex-2-en-1,4-dione and treatments in the presence of S9-mix used the pre-incubation method. The test chemical elicited evidence of cytotoxicity in the form of background lawn thinning or marked reduction of the number of revertants in experiment 1 at 1 000 and/or 5 000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix and in experiment 2 at 2 500 and/or 5 000 µg/plate in strain TA102 in the absence and presence of S9-mix. Thus, the study design complied with current recommendations and an acceptable highest concentration was achieved. There was no evidence of any mutagenic effect induced by 2,6,6-trimethylcyclohex-2-en-1,4-dione in any of the strains, either in the absence or presence of S9-mix.

Summary of the bacterial reverse mutation assay for all the substances is reported in Table 10.

Micronucleus Assay

β-Ionone [FL-no: 07.008]

β-Ionone [FL-no: 07.008] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system. Cells were stimulated for 48 hours with phytohaemagglutinin (PHA) to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 30, 50 or 60 µg/ml of β-ionone in the absence of S9-mix and 0, 80, 100 or 120 µg/ml in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 52 % and 59 %, respectively.

In a parallel assay, cells were treated for 24 hours with 0, 5, 15 and 17.50 µg/ml of β-ionone in the absence of S9-mix with no recovery period. The highest concentration induced 58 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable levels of cytotoxicity were achieved at the highest concentrations used in all parts of the study. Treatment of cells with β-ionone for 3 hours with a 21-hour recovery period showed an increase in the frequency of micronucleated binucleated (MNBN) cells in one single replicate at concentrations of 30 and 120 µg/ml (0.9 % and 1.5 %, respectively) in the absence and presence of S9-mix, respectively. At 30 µg/ml, the lowest concentration tested in the absence of S9-mix, the increase in the frequency of MNBN cells was slightly above the 95 % confidence interval of the historical control range (0.2–0.8 %). In addition, in the presence of S9-mix, one replicate of the lowest concentration tested (80 µg/ml) had an increase in the frequency of MNBN cells at the upper limit of the 95 % confidence interval of the historical control range (0.10–1.10 %) but did not reach statistical significance. To ensure that these single occurrences are random an additional 1 000 binucleate cells were scored from the concurrent controls, 80 and 120 µg/ml cultures. The scoring of further cells resulted in overall mean frequencies of MNBN cells that were not significantly different from concurrent controls and fell below the upper 95 % confidence interval of the normal control range (recalculated due to change of stain), and therefore showed that the earlier increases were due to chance. It was concluded that β-ionone [FL-no: 07.008] did not induce micronuclei up to toxic concentrations when assayed in cultured human peripheral lymphocytes for 3 + 21 hours in the absence and presence of S9-mix or when incubated for 24 + 0 hours in the absence of S9-mix (Stone, 2011a).

Maltol [FL-no: 07.014]

Maltol [FL-no: 07.014] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system (Whitwell, 2012). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 400, 800 or 1 262 µg/ml of maltol, the last being equivalent to 10 mM, in the absence and presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 24 % and 19 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 125, 200 and 300 µg/ml of maltol in the absence of S9-mix with no recovery period. The highest concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment, and 1 000 binucleate cells per replicate (i.e. 2 000 cells per concentration) were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable highest concentrations, either 10 mM or 50–60 % toxicity, were achieved in all parts of the study. A statistically significant increase in the occurrence of MNBN cells was observed following 3 + 21 hours treatment in the presence of S9-mix at the two highest concentrations scored. Statistically significant and concentration-dependent increases in MNBN cells were seen in the 3 + 21 hours treatment groups in the absence of S9-mix, but it was noted that the increases at the two highest concentrations scored exceeded historical control ranges in only one of the two replicate cultures. No increases were observed in the frequency of MNBN cells in those that had received continuous (24 + 0 hours) treatment, but due to the cytotoxicity of maltol, lower concentrations were analysed. To further investigate these observations, fluorescence *in situ* hybridisation (FISH) analysis using human pan-centromeric probes was conducted to assess whether the mechanism of action could be attributed to chromosome loss (aneuploidy) or chromosome breakage (clastogenicity). Slides were prepared from the two highest concentrations (800 and 1 262 µg/ml) in the absence and presence of S9-mix. The FISH analysis revealed that following maltol treatment the majority (69–76 %) of micronuclei did not contain a centromere. The Panel concluded that maltol induced micronuclei *in vitro* in cultured human peripheral blood lymphocytes in the presence of rat liver metabolic activation (S9-mix) via a clastogenic mechanism of action (Whitwell, 2012). However, the Panel considered that the results observed in the absence of S9-mix were equivocal because of the fact that the increases observed (which were statistically significantly different from concurrent solvent control) were not reproduced in replicate cultures.

β-Damascone [FL-no:07.083]

β-Damascone (purity: 95 %) was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence or absence of rat S9 fraction as an *in vitro* metabolising system (Stone, 2012). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with concentrations ranging from 2 to 30 µg/ml. For the treatment of 3 hours with a 21-hour recovery period, the concentrations of β-damascone at 8, 16 and 22 µg/ml or at 12, 16, 18 µg/ml were retained for micronuclei (MN) numeration, in the absence or in the presence of S9-mix respectively. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 59 % and 51 %, respectively. Thus, the study design complies with OECD Guideline 487 and follows GLP principles.

In a parallel assay, cells were treated for 24 hours (with no recovery period) in the absence of S9-mix with concentrations ranging from 1 to 15 µg/ml, and the concentrations of 6, 8 and 9 µg/ml of β-damascone were retained for MN numeration. The highest concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and 1 000 binucleate cells per replicate were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487, 2010), and acceptable levels of cytotoxicity were achieved at the highest concentrations used in all parts of the study.

Treatment of cells with β-damascone for 3 + 21 hours in the presence of S9-mix showed a statistically significant concentration-dependent increase in the induction of MNBN cells with 0.55, 2.10 and 2.70 % MNBN cells versus 0.35 % in the concurrent control and 0.1 to 1.1 % for the historical controls.

Treatment of cells with β-damascone for 3 + 21 and 24 + 0 hours in the absence of S9-mix resulted in sporadic increases in MNBN frequency. These increases were only observed in single replicates and were not concentration-related. Therefore, the effect of β-damascone was further investigated through the scoring of additional cells (2 more replicates of 1000 cells each) from the affected concentrations and concurrent controls.

Treatment of cells, in the absence of S9-mix, for 3 + 21 hours induced a statistically significant increase in the frequency of MNBN cells at 8 and 22 µg/ml (0.80 % and 0.93 %, respectively) compared with concurrent control (0.38 %), but not at the mid-dose of 16 µg/ml (0.53 % MNBN cells). The frequency of MNBN cells exceeded the historical controls (0.2–0.8 %) in 3 out of 4 replicates at the highest concentration tested (22 µg/ml). Treatment of cells for 24 hours with no recovery period in the absence of S9-mix showed a statistically significant increase in the frequency of MNBN cells at the mid-dose of 8 µg/ml (0.95 % MNBN cells) when compared with concurrent control (0.40 %) with no correlation to concentration. The frequency of MNBN cells exceeded the historical controls (0–1.1 %) in only one replicate at 8 µg/ml.

The authors considered that this result reaffirmed the sporadic nature of the induction of MNBN cells in the absence of S9-mix. It was concluded that the treatment with β-damascone for 3 + 21 hours or 24 + 0 hours (in the absence of S9-mix) induced sporadic increases in MNBN cells when compared with concurrent controls and not concentration-related; therefore, the results were considered equivocal. In the same test system, β-damascone induced micronuclei in cultured human peripheral blood lymphocytes following 3 + 21 hours treatment in the presence of S9-mix (Stone, 2012). The Panel noted that after the new reading of slides the increase in the frequency of MNBN cells was still statistically significant even at weak cytotoxic levels.

Therefore, the Panel concluded that β-damascone is genotoxic in the *in vitro* micronucleus assay on human lymphocytes with metabolic activation and equivocal without metabolic activation.

Nootkatone [FL-no: 07.089]

Nootkatone [FL-no: 07.089] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Stone, 2011b). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 50, 70 or 80 µg/ml of nootkatone in the absence of S9-mix and 0, 160, 180 and 185 µg/ml in the presence of S9-mix, respectively. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 60 % and 58 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 10, 15, 22 and 24 µg/ml of nootkatone in the absence of S9-mix with no recovery period. The highest concentration induced 62 % cytotoxicity. There were 2 replicate cultures per treatment and 1 000 binucleate cells per replicate (i.e. 2 000 cells per dose) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487) and acceptable levels of cytotoxicity were achieved at the highest concentrations used in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical control ranges for all treatments with nootkatone in the presence or absence of S9-mix metabolic activation (Stone, 2011b).

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

2,6,6-Trimethylcyclohex-2-en-1,4-dione was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Lloyd, 2011). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 500, 1 000 or 1 522 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix and 0, 1 000, 1 250 and 1 522 µg/ml in the presence of S9-mix, the highest concentration being equivalent to 10 mM. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 3 % and 9 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 300, 420 and 550 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix with no recovery period. The highest concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and

1 000 binucleate cells per replicate (i.e. 2 000 cells per concentration) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487), and acceptable highest concentrations, either 10 mM or 50–60 % toxicity, were achieved in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical ranges for all 2,6,6-trimethylcyclohex-2-ene-1,4-dione treatments in the presence or absence of S9-mix metabolic activation (Lloyd, 2011).

The results of *in vitro* micronucleus studies are summarised in Table 10.

2.5.3. Genotoxicity *in vivo* data

In vivo Combination Assay (comet + micronucleus tests)

Since no positive results were seen in either the bacterial mutation assay or *in vitro* micronucleus tests with β-ionone [FL-no: 07.008], nootkatone [FL-no: 07.089] or 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], no *in vivo* follow-up testing was required. To address the effects seen in the *in vitro* micronucleus assay with maltol [FL-no: 07.014] and β-damascone [FL-no: 07.083] a combination assay comprising a liver comet assay and an *in vivo* micronucleus assay in rats, after oral application, was performed to further assess the genotoxic potential for both substances. The results are summarised in Table 11.

Maltol [FL-no: 07.014]

Maltol was evaluated in an *in vivo* bone marrow micronucleus assay and liver comet assay in male Han Wistar (HsdHan:WIST) rats, with 6 rats per dose group (Beevers, 2013a). The rats were administered 3 doses of 70, 350 and 700 mg/kg bw of maltol by oral gavage at time 0, 24 and 45 hours. Rats were killed and sampled at 48 hours post the initial dose. The maximum tolerable dose was estimated to be 700 mg/kg bw/day based on a range-finding study where maltol was tested at 360, 500, 700, 1 000, 1 500 and 2 000 mg/kg bw/day. Clinical observations (piloerection, ataxia, bradypnoea) and mortalities were observed at doses of 1000 mg/kg bw/day and greater. For the micronucleus assay 2 000 polychromatic erythrocytes (PCE) per rat were scored. The negative control had a normal, low frequency (0.11 %) of micronucleated polychromatic erythrocytes (MNPCEs) and a ratio of 53.7 % PCE. The positive control group resulted in a significant increase in MNPCEs (1.58 %) accompanied by some bone marrow toxicity (29.57 % PCE). Although an individual rat in the 700 mg/kg maltol dose group showed a frequency of 9 MNPCEs, which resulted in significant heterogeneity in this dose group, this was considered an outlying data point because the other 5 rats in the group were exhibiting normal control-level MNPCE frequencies (Beevers, 2013a). Overall, the results showed that there were no statistically significant increases in micronucleus frequency for any dose group after oral treatment with maltol when compared with the vehicle control group. However, in the main experiment, at the dose levels selected, no clinical signs and bone marrow toxicity were observed in any animal in the maltol-treated groups, which may reflect the possibility that the bone marrow and liver were not exposed.

In order to clarify this issue, the Panel requested plasma analysis for proof of exposure. Plasma was obtained from two satellite groups of male animals (3 + 3 animals) dosed with maltol by oral gavage at 700 mg/kg bw/day, during conduction of the main study (Beevers, 2013a). Plasma obtained from 0.5 ml blood drawn from the jugular vein from each animal was frozen in the event that analysis for proof of exposure and toxicokinetics were required. All doses of maltol were given as three administrations, at 0, 24 and 45 hours. Three samples of plasma were obtained from one group of animals at 0.5, 2 and 8 hours and three samples from the other group at 1, 4 and 24 hours from the last administration. Analysis of maltol in plasma was performed using a gas chromatography with mass selective detection (GC-MSD) method. From an analytical point of view, the Panel considered the employed approach, which was based on the use of ethylmaltol as an internal standard, as sufficient. Results showed a marked inconsistency between sampling times and animals. In samples collected at 0.5, 2 and 8 hours from last administration maltol was found in 2 out of 3 satellite animals at plasma concentrations of 265–283 µg/ml after 0.5 and 2 hours, but no longer detectable after 8 hours. In the plasma of the third animal maltol was not detectable at any time. On the other hand, in samples from another animal group (n = 3) collected at 1, 4 and 24 hours from last administration, maltol was

found at levels of 75–106 µg/l after 1 hour in all 3 animals and no longer detectable after 4 and 24 hours (Mallinson and Hough, 2014). The authors concluded that the results obtained provided evidence that maltol is present in plasma shortly after dosing. However, the Panel did not agree with this conclusion and it considered the results of the bioanalytical study as inconclusive.

In the combined comet assay, livers of rats were removed at 48 hours after the first dose (i.e. 3 hours after the final dose), cut into small pieces and forced through a bolting cloth. Single cell suspensions were embedded in low melting point agarose on slides and lysed. The DNA was unwound and subjected to electrophoresis at pH > 13 and then neutralised according to standard techniques. For each animal, 100 cells (50 cells/slide from 2 slides) were scored for comets (tail intensity and tail moment) using commercial image analysis equipment.

The comet assay did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis and the groups treated with maltol showed mean percentage tail intensities and tail moments that were similar to vehicle controls and fell within historical control ranges. The positive control group treated with ethyl methanesulphonate showed significant increases in both parameters (Beevers, 2013a).

Considering that maltol has been shown to induce micronuclei in mouse bone marrow after intraperitoneal injection (Hayashi et al., 1988), the Panel concludes that negative findings observed in the combined bone marrow micronucleus test and comet assay in the liver of treated rats could not rule out the concern for genotoxicity for maltol since the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

β-Damascone [FL-no:07.083]

A combined *in vivo* micronucleus assay and comet assay was performed after oral application of β-damascone (purity: 95.6 %) to further assess the genotoxic potential of β-damascone and damascones more generally. The results are summarised in Table 11. β-Damascone was evaluated in an *in vivo* bone marrow micronucleus assay and liver and duodenum comet assay in groups of 6 male Han Wistar (HsdHan:WIST) rats per dose group (Beevers, 2013c). Based on a range-finding study, 500 mg/kg/day was considered an appropriate estimate of the maximum tolerated dose (MTD) because doses of 750 mg/kg/day and greater induced moderate to severe clinical signs of toxicity, which included piloerection, decreased activity, hunched posture and abnormal breathing. The rats were administered 3 doses of 125, 250 and 500 mg/kg bw of β-damascone by oral gavage at time 0, 24 and 45 hours. The rats were sacrificed and sampled at 48 hours post the initial dose.

Animals administered β-damascone showed clear findings during pathological analysis. Hepatocyte vacuolation was present in animals given 500 mg/kg/day, and was characterised by scattered, occasionally shrunken hepatocytes with perinuclear cytoplasmic eosinophilia and peripheral cytoplasmic vacuolation. Single cell necrosis was present in a single animal given 500 mg/kg/day. Single cell necrosis was characterised by death of individual hepatocytes throughout the liver, with limited inflammatory cell involvement. There was a dose-related reduction in the level of glycogen vacuolation in animals given 250 or 500 mg/kg/day. Glycogen vacuolation was characterised by generally perinuclear, clear, variably sized, indistinctly defined, vacuoles. Finally, increased mitosis was present in animals from all groups given β-damascone. The greatest severity was present in animals given 250 mg/kg/day, and the lowest incidence was present in animals given 500 mg/kg/day. Increased mitosis was characterised by an increase, above the normal low background incidence, of mitotic figures within the liver parenchyma. Collectively, these findings indicate that the test animals were systemically exposed to β-damascone.

The negative control had a 0.11 % average rate of MNPCE and a ratio of 50.2 % PCE; the 125 mg/kg β-damascone treatment group had a MNPCE rate of 0.09 % and PCE ratio of 49.17 %; the 250 mg/kg treatment group had a 0.09 % MNPCE rate and 52.30 % PCE ratio; the 500 mg/kg treatment group showed 0.06 % MNPCEs and 37.63 % PCE ratio. The positive control group resulted in 1.54 % MNPCEs and a 43.17 % PCE ratio (Beevers, 2013c). The group mean frequencies observed were similar to concurrent vehicle controls for all dose groups and also were within the historical control values (mean: 0.12 %). There was a reduction in PCE ratio at the highest dose level indicating bone marrow toxicity, which demonstrates target organ exposure. These results showed that there was no statistically significant increase in micronuclei induced with β-damascone under these test conditions when compared with the negative control group. In addition, there were no statistically significant

differences among erythrocyte parameters examined in this study. It was concluded that β-damascone did not induce micronucleated erythrocytes in rat bone marrow cells following administration by oral gavage.

The comet assay in the liver tissue did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis. Hepatocytes of rats dosed with β-damascone were evaluated for percentage tail intensities and tail moments (± standard error of the mean, SEM); the 125 mg/kg β-damascone group had 2.45 ± 0.13 % tail intensity and 0.27 ± 0.02 % tail moment; the 250 mg/kg group had 2.99 ± 0.31 % tail intensity and 0.33 ± 0.03 tail moment; the 500 mg/kg group had 2.93 ± 0.24 % tail intensity and 0.31 ± 0.03 tail moment, which were similar to concurrent vehicle controls (tail intensity of 2.67 ± 0.26 % and 0.29 ± 0.03 tail moment) and fell within the testing laboratories historical control range for vehicle controls (0.3–8.15 % tail intensity and 0.04–0.81 tail moment). The comet arm of this study confirms that β-damascone did not induce DNA damage in the liver under the conditions of this study (Beevers, 2013c).

In a satellite study the slides from the duodenum tissue samples collected in the above study (Beevers, 2013c) were analysed for comet tailing effects (Beevers, 2013b). Duodenum cells of rats dosed with β-damascone were evaluated for percentage tail intensities and tail moments (± standard error of the mean, SEM); the 125 mg/kg β-damascone group had 2.01 ± 0.43 % tail intensity and 0.32 ± 0.03 % tail moment; the 250 mg/kg group had 1.47 ± 0.15 % tail intensity and 0.16 ± 0.02 tail moment; the 500 mg/kg group had 2.03 ± 0.19 % tail intensity and 0.19 ± 0.02 tail moment, which were similar to concurrent vehicle controls (tail intensity of 2.24 ± 0.43 % and 0.23 ± 0.04 % tail moment) and fell within the testing laboratories historical control range for vehicle controls (0.3–8.15 % tail intensity and 0.04–0.81 tail moment). The duodenum comet arm of this study confirms that β-damascone did not induce DNA damage in the duodenum under the conditions of this study. The vehicle control data were within historical control ranges (95 % reference range: 0.77 to 8.32 % for tail intensity and 0.08 to 1.15 % for tail moment) and the positive control induced a clear increase in DNA damage. The study was therefore confirmed as valid. There was no evidence of duodenum toxicity as would be suggested by increases in clouds or halo cells.

The percentage tail intensity and tail moment at all dose levels were very similar to the concurrent vehicle control, thus confirming there is no test article-related DNA damage. The additional tissue sample analysis for comet tailing showed a negative result for this study (Beevers, 2013b).

The results from the combined *in vivo* micronucleus induction study and comet assay show that orally administered β-damascone did not induce micronucleated erythrocytes in rat bone marrow cells nor genotoxic events in liver and duodenum of rats.

2.5.4. Conclusion

Flavouring Group Evaluation 213 concerned 26 substances, corresponding to subgroup 2.7 of FGE.19 (see Table 4). Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-nos: 02.106, 09.305 and 09.525].

For 11 substances [FL-nos: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168] the Panel have ruled out concerns regarding genotoxicity in FGE.213.

In FGE.213Rev1, new data have been evaluated for the representative of the remaining substances. More specifically, data for β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], maltol [FL-no: 07.014], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]. All these studies are fully compliant with current guidelines, and stand in contrast to earlier studies previously evaluated in FGE.213.

The combined evidence from *in vitro* and *in vivo* genotoxicity data for the selected representative substances β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] does not indicate a genotoxic potential. Therefore, these substances and the nine substances that they represent [FL-nos: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305] could be evaluated through the Procedure.

For maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525], the Panel concluded that the concern for genotoxicity could not be ruled out.

3. Assessment

3.1. Additional data evaluated by the Panel in FGE.213Rev2

3.1.1. Plasma bioanalysis

Since data provided to prove the systemic exposure to maltol of the animals tested in the combined bone marrow micronucleus test and comet assay in liver (Beevers, 2013a) were considered inconclusive in FGE.213Rev1, the Flavour Industry has submitted a new plasma bioanalysis (Beevers, 2015).

Six male Han Wistar rats were dosed at 700 mg maltol/kg bw/day (determined previously as an estimate of the MTD), using the same dosing regimen employed in the combined micronucleus test and comet assay (Beevers, 2013a).

Maltol was prepared as a suspension 0.5 % (w/v) in aqueous methylcellulose and administered via oral gavage at: 0 (day 1), 24 (day 2) and 45 (day 3) hours. Whole blood was collected at 0.5, 1, 2 and 3 hours after dosing on day 3. Plasma was isolated and analysed using GC–MSD. Ethyl maltol was used as an internal standard.

Detectable levels of maltol were found in all plasma samples isolated at 0.5, 1 and 2 hours after dosing. Peak plasma levels of maltol were seen in the majority of animals at 0.5 hours after dose administration. The concentration of maltol detected in plasma was different between the animals of 2 separate cages (treated with the same dose) and the authors of the study did not identify any technical reasons that could account for this difference. However, it was concluded that data demonstrate the presence of maltol in blood and that accordingly the bone marrow could be considered exposed.

3.1.2. Additional *in vitro* data on maltol

An *in vitro* chromosomal aberration assay on maltol (not available before) is considered in the present revision of FGE.213. Maltol (purity 99 %) was tested in CHL fibroblast cell line at three concentrations: 25, 50 and 75 µg/ml. Cells were harvested for chromosomal preparations after 24 hours or 48 hours from the beginning of the treatment; metabolic activation was not included. Structural chromosomal aberrations were observed at the 2 highest concentrations tested after both 24 hours and 48 hours of treatment. These increases were concentration related, polyploidy was not observed (Ishidate, 1988). The result obtained in this study is consistent with the clastogenic effect of maltol observed in the study by Whitwell (2012), described in Section 2.5.2.

3.1.3. Additional information on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127]

In FGE.213Rev1, the Panel concluded that the genotoxicity concern for *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] could be ruled out based on available genotoxicity data on structurally related substances and consequently could be evaluated through the Procedure in FGE.57Rev1.

During the evaluation of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] through the Procedure, the Panel noted that the chemical structure of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] is actually more closely related to the structure of pulegone than to the structures used for the read-across approach in FGE.213Rev1.

New information was found on genotoxicity and carcinogenicity of pulegone, based on which additional data are expected to be provided by the applicant. At present, the data available on pulegone and on the structurally related substance *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] do not rule out the concern for genotoxicity and carcinogenicity. Therefore *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] will be re-evaluated pending the submission of additional genotoxicity data.

4. Conclusion

The Panel considered the available genotoxicity studies on maltol. An *in vivo* study in mice (by intraperitoneal route) showed that maltol induced a statistically significant increase in the incidence of micronuclei in bone marrow cells. Since maltol is intended to be used as food flavouring agent, the *in vivo* study performed by gavage (combined micronucleus test and comet assay in rats) was considered more relevant, but in the previous revision of that opinion, this study did not allow conclusions on the genotoxicity of maltol to be made because exposure of target tissue was not demonstrated. Therefore, the Panel requested to investigate the systemic exposure of animals to maltol.

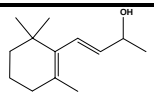
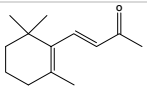
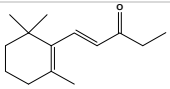
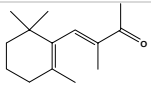
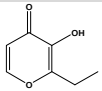
The Panel considered the new plasma bioanalysis for maltol and concluded that, based on the new data provided, it now seems justifiable to assume that the animals were systemically exposed to maltol and that the bone marrow was exposed in the *in vivo* micronucleus assay.

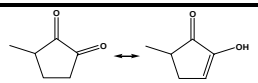
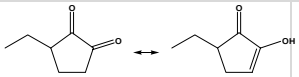
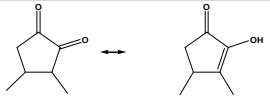
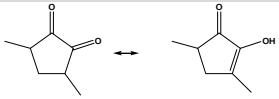
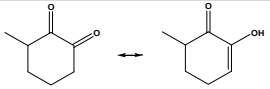
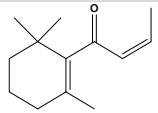
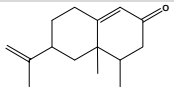
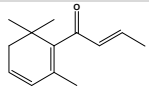
Therefore, the negative result of the *in vivo* micronucleus assay can be considered reliable and, accordingly, the concern for genotoxicity for maltol [FL-no: 07.014] and for maltyl isobutyrate [FL-no: 09.525] in food is ruled out; both substances were evaluated by JECFA before 2000 and no EFSA consideration is required.

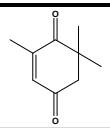
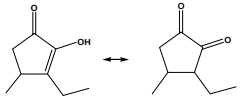
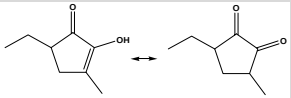
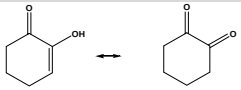
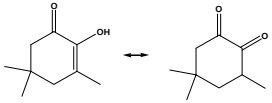
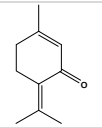
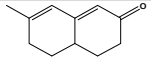
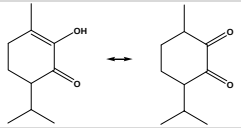
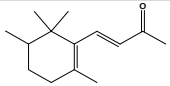
The Panel reconsidered the available data on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] based on new data for the structurally related substance pulegone, and concluded that additional genotoxicity data are needed for [FL-no: 07.127] to rule out the concern for genotoxicity.

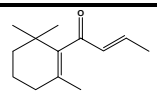
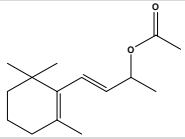
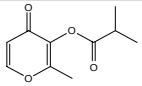
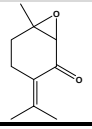
4.1. Summary of Specification for Substances in FGE.213 (JECFA 1998, 2000, 2005a, b, 2009b)

Table 4: Specification summary of the substances in FGE.213

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
02.106 **392**	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		3625 – 22029-76-1	Liquid $C_{13}H_{22}O$ 194.32	– –	107 (4 hPa) – IR 92 %	1.499 0.927–0.933
07.008 **389**	β-Ionone		2595 142 14901-07-6	Liquid $C_{13}H_{20}O$ 192.30	Insoluble 1 ml in 3 ml 70 % alcohol	239 – IR 95 %	1.517–1.522 0.940–0.947
07.010 **399**	Methyl-β-ionone		2712 144 127-43-5	Liquid $C_{14}H_{22}O$ 206.33	– –	238–242 – IR 88 %	1.503–1.508 0.930–0.935
07.014 **1480**	Maltol		2656 148 118-71-8	Solid $C_6H_6O_3$ 126.11	Very slightly soluble Soluble	– 159–162 NMR 98 %	n.a. n.a.
07.041	β-Isomethylionone		4151 650 79-89-0	Solid $C_{14}H_{22}O$ 206.32	– Freely soluble	334 62 – 95 %	n.a. n.a.
07.047 **1481**	Ethyl maltol		3487 692 4940-11-8	Solid $C_7H_8O_3$ 140.14	Soluble Soluble	– 89–93 NMR 99 %	n.a. n.a.

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.056 **418**	3-Methylcyclopentan-1,2-dione		2700 758 80-71-7	Solid $C_6H_8O_2$ 112.13	1 g in 72 ml water 1 g in 5 ml 90 % alcohol	– 104–108 IR 95 %	– –
07.057 **419**	3-Ethylcyclopentan-1,2-dione		3152 759 21835-01-8	Solid $C_7H_{10}O_2$ 126.16	Miscible –	78–80 (5 hPa) 36–43 IR 90 %	1.47–1.48 (25°) 1.060–1.066
07.075 **420**	3,4-Dimethylcyclopentan-1,2-dione		3268 2234 13494-06-9	Solid $C_7H_{10}O_2$ 126.16	– –	66 (1 hPa) 68–72 IR 98 %	– –
07.076 **421**	3,5-Dimethylcyclopentan-1,2-dione		3269 2235 13494-07-0	Solid $C_7H_{10}O_2$ 126.16	Insoluble –	– 87–93 MS 98 %	– –
07.080 **425**	3-Methylcyclohexan-1,2-dione		3305 2311 3008-43-3	Solid $C_7H_{10}O_2$ 126.16	Insoluble –	69–72 (1 hPa) 57–63 IR 98 %	– –
07.083 **384**	β-Damascone		3243 2340 23726-92-3	Liquid $C_{13}H_{20}O$ 192.30	– 1 ml in 10 ml 95 %	67–70 – IR 90 %	1.496–1.501 0.934–0.942 (20°)
07.089 **1398**	Nootkatone		3166 11164 4674-50-4	Liquid $C_{15}H_{22}O$ 218.35	Slightly soluble Soluble	73–103 (1 hPa) – NMR 93 %	1.510–1.523 1.003–1.032
07.108 **387**	β-Damascenone		3420 11197 23696-85-7	Liquid $C_{13}H_{18}O$ 190.28	– 1 ml in 10 ml 95 % alcohol	60 – IR 98 %	1.508–1.514 0.945–0.952 (20°)

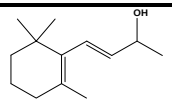
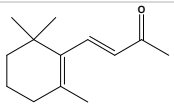
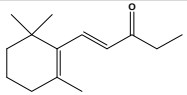
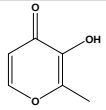
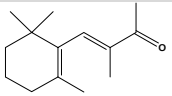
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.109 **1857**	2,6,6-Trimethylcyclohex-2-en-1,4-dione		3421 11200 1125-21-9	Solid $C_9H_{12}O_2$ 152.2	Slightly soluble Soluble	222 23–28 IR NMR 98 %	n.a. n.a.
07.117 **422**	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		3453 11077 42348-12-9	Liquid $C_8H_{12}O_2$ 140.18	Slightly insoluble Miscible	– – NMR 99 %	1.481–1.487 1.055–1.061
07.118 **423**	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		3454 11078 53263-58-4	Liquid $C_8H_{12}O_2$ 140.18	Slightly soluble Soluble	– – NMR 99 %	1.478–1.484 1.053–1.060
07.119 **424**	2-Hydroxycyclohex-2-en-1-one		3458 11046 10316-66-2	Solid $C_6H_8O_2$ 112.13	Soluble Soluble	53 (3 hPa) 35–38 IR 99.3 %	– –
07.120 **426**	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		3459 11198 4883-60-7	Solid $C_9H_{14}O_2$ 154.21	Slightly soluble Soluble	90–100 (20 hPa) 88 IR 99 %	– –
07.127 **757**	*p*-Mentha-1,4(8)-dien-3-one		3560 11189 491-09-8	Liquid $C_{10}H_{14}O$ 150.22	Insoluble Miscible	233 – MS 95 %	1.472–1.478 0.976–0.983
07.136 **1405**	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		3715 – 34545-88-5	Solid $C_{11}H_{14}O$ 162.23	Insoluble Soluble	n.a. 36–37 IR 99 %	n.a. n.a.
07.168 **2038**	2-Hydroxypiperitone		4143 – 490-03-9	Solid $C_{10}H_{16}O_2$ 168.24	Slightly soluble Freely soluble	233 82 NMR MS 98 %	n.a. n.a.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		– – 79-70-9	Liquid $C_{14}H_{22}O$ 206.33	Practically insoluble or insoluble Freely soluble	108 (2 hPa) – MS 95 %	1.515–1.521 0.943–0.949

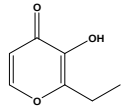
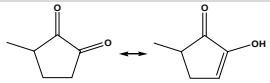
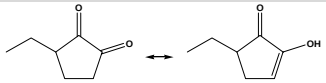
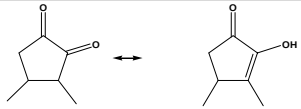
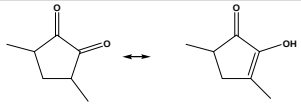
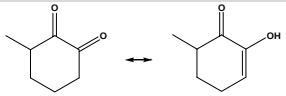
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		3243 2340 23726-91-2	– – –	– –	– – – 90 %	– –
09.305 **1409**	β-Ionyl acetate		3844 10702 22030-19-9	Liquid $C_{15}H_{24}O_2$ 236.35	Insoluble Soluble	120 (3 hPa) – NMR 92 %	1.474–1.484 0.934–0.944
09.525 **1482**	Maltyl isobutyrate		3462 10739 65416-14-0	Liquid $C_{10}H_{12}O_4$ 196.20	Insoluble Soluble	100 (0.01 hPa) – IR 96 %	1.493–1.501 1.140–1.153
16.044 **1574**	Piperitenone oxide		4199 10508 35178-55-3	Solid $C_{10}H_{14}O_2$ 166.22	Soluble Soluble	– 25 NMR MS 95 %	n.a. n.a.

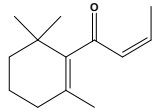
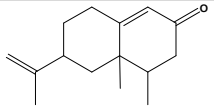
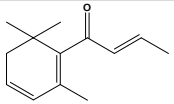
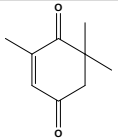
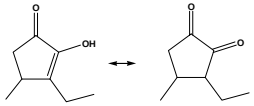
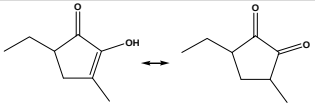
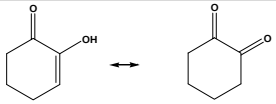
n.a.: not applicabile; (-): data not reported
(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1 013.25 hPa, if not otherwise stated.
(d): At 20 °C, if not otherwise stated.
(e): At 25 °C, if not otherwise stated.

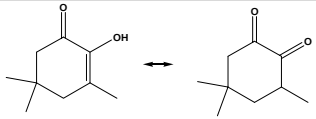
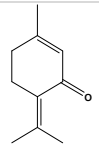
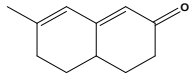
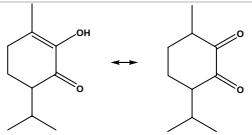
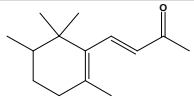
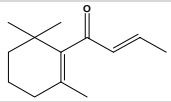
4.2. Summary of Safety Evaluation Applying the Procedure (JECFA 1999, 2001, 2006a, b, 2009a)

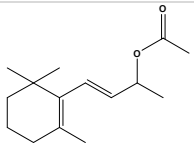
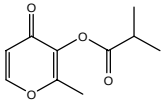
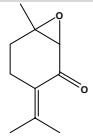
Table 5: Summary of safety evaluation applying the procedure

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
02.106 **392**	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		0.73 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.008 **389**	β-Ionone		130 100	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.010 **399**	Methyl-β-ionone		5.4 0.2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.014 **1480**	Maltol		3 060 2 898	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev2, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.041	β-Isomethylionone		0.011	Not evaluated by the JECFA		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.047 **1481**	Ethyl maltol		1 580 6 692	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.83Rev1. No safety concern at the estimated level of intake based on the MSDI approach.
07.056 **418**	3-Methylcyclopentan-1,2-dione		570 710	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.057 **419**	3-Ethylcyclopentan-1,2-dione		32 23	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.075 **420**	3,4-Dimethylcyclopentan-1,2-dione		30 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.076 **421**	3,5-Dimethylcyclopentan-1,2-dione		35 29	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.080 **425**	3-Methylcyclohexan-1,2-dione		1.3 8	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.083 **384**	β-Damascone		37 10	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.089 **1398**	Nootkatone		130 20	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.108 **387**	β-Damascenone		73 5	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.109 **1857**	2,6,6-Trimethylcyclohex-2-en-1,4-dione		50	Class II No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.09Rev5.
07.117 **422**	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		ND 0.17	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.118 **423**	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		ND 0.38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.119 **424**	2-Hydroxycyclohex-2-en-1-one		0.049 0.76	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA

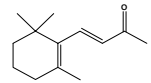
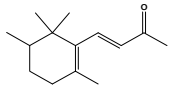
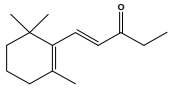
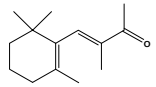
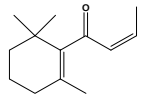
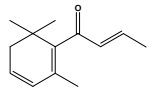
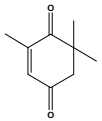
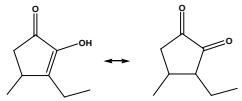
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
						before 2000. No EFSA consideration required.
07.120 **426**	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		1.2 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.127 **757**	*p*-Mentha-1,4(8)-dien-3-one		0.012 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1 and FGE.213Rev2, genotoxicity concern could not be ruled out. Additional data are requested.
07.136 **1405**	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		ND 0.04	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.168 **2038**	2-Hydroxypiperitone		0.0012	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.11Rev2. No safety concern at the estimated level of intake based on the MSDI approach.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		0.012	Class I No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		100	No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated

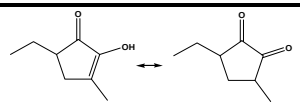
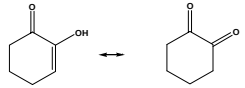
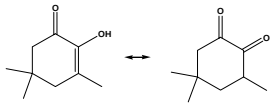
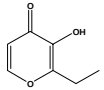
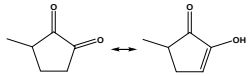
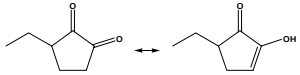
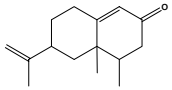
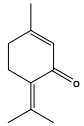
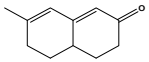
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
						using the Procedure in FGE.12Rev5.
09.305 **1409**	β-Ionyl acetate		ND 9	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.73Rev3. MSDI based on USA production figure.
09.525 **1482**	Maltyl isobutyrate		20 38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev2, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
16.044 **1574**	Piperitenone oxide		0.012 0.2	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, additional genotoxicity data required. The substance is not supported by the Flavour Industry any longer. No further evaluation.

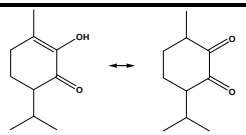
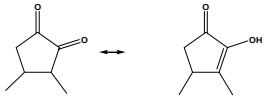
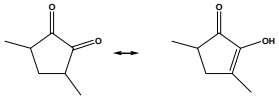
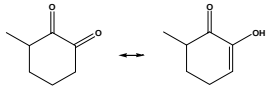
ND: no data
(a): EU MSDI: Amount added to food as flavour in (kg/year) x 10E9/(0.1 × population in Europe (= 375 × 10E6) × 0.6 × 365) = μg/capita/day
(b): Thresholds of concern: class I = 1800 μg/person/day, class II = 540 μg/person/day, Class III = 90 μg/person/day
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot
(d): No safety concern based on intake calculated by the MSDI approach of the named compound
(e): Data must be available on the substance or closely related substances to perform a safety evaluation

4.3. (Q)SAR Predictions on Mutagenicity in Five Models for 22 Ketones from Subgroup 2.7

Table 6: (Q)SAR predictions on mutagenicity for 22 alicyclic ketones from subgroup 2.7

FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.008 389	β-Ionone		NEG	NEG	NEG	NEG	EQU
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		NEG	NEG	NEG	NEG	EQU
07.010 399	Methyl-β-ionone		NEG	NEG	OD	OD	EQU
07.041	β-Isomethylionone		NEG	EQU	NEG	NEG	NEG
07.083 384	β-Damascone		OD	NEG	OD	OD	EQU
07.108 387	β-Damascenone		OD	NEG	OD	OD	EQU
07.109	2,6,6-Trimethylcyclohex-2-en-1,4-dione		OD	NEG	OD	NEG	EQU
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		OD	NEG	NEG	OD	NEG

FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		OD	NEG	NEG	NEG	NEG
07.119 424	2-Hydroxycyclohex-2-en-1-one		OD	OD	NEG	OD	NEG
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		OD	NEG	NEG	OD	NEG
07.014 1480	Maltol		OD	OD	NEG	OD	POS
07.047 1481	Ethyl maltol		OD	OD	NEG	OD	POS
07.056 418	3-Methylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.057 419	3-Ethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.089 1398	Nootkatone		OD	NEG	NEG	NEG	POS
07.127 757	*p*-Mentha-1,4(8)-dien-3-one		OD	NEG	OD	NEG	NEG
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		OD	NEG	NEG	NEG	OD

FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.168 -	2-Hydroxypiperitone		OD	NEG	NEG	NEG	NEG
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	NEG	NEG
07.080 425	3-Methylcyclohexan-1,2-dione		OD	NEG	NEG	OD	NEG

OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological etc.
(a): Structure group 2.7: α,β-unsaturated ketones.
(b): Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD*: out of domain).
(c): MultiCASE Ames test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(d): MultiCASE Mouse Lymphona test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(e): MultiCASE Chromosomal aberration in CHO (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(f): MultiCASE Chromosomal aberration in CHL (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

4.4. Genotoxicity data (*in vitro*) considered by the Panel in FGE.213

Table 7: Genotoxicity (*in vitro*)

Chemical name [FL-no]	Test system	Test object	Concentration	Reported result	Reference	Comments [(d)]
β-Ionone [07.008]	Gene mutation (preincubation)	*S. typhimurium* TA98, TA100, TA1535, TA1537	1–180 µg/plate	Negative [(a)]	Mortelmans et al., 1986	Valid.
	Gene mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3 mmol/plate	Negative [(a)]	Florin et al., 1980	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
3-Methylcyclopentan-1,2-dione [07.056]	Reverse mutation	*S. typhimurium* TA1535	10 000 µg/plate	Negative [(b)]	Heck et al., 1989	Validity cannot be evaluated (result not reported in detail).
	Unscheduled DNA synthesis	Rat hepatocytes	500 µg/plate	Negative [(b)]	Heck et al., 1989	Validity cannot be evaluated (result not reported in detail).
Maltol [07.014]	Reverse mutation	*S. typhimurium* TA100	4.44 µmol/plate (560 µg/plate)	Negative [(c)]	Kim et al., 1987	Insufficient validity (only one concentration was tested with only one bacterial strain without metabolic activation). The main purpose of the study was to investigate antimutagenic effects.
	Reverse mutation	*S. typhimurium* TA98 and TA100	Up to 3 mg/plate (3 000 µg/plate)	Positive [(a)]	Bjeldanes and Chew, 1979	Valid.
	Reverse mutation	*S. typhimurium* TA92, TA98, TA100 and TA104	1.5 to 11 µmol/plate (189 to 1 387 µg/plate)	Negative	Gava et al., 1989	Limited validity (data not reported in detail).
	Reverse mutation	*S. typhimurium* TA1535, TA98, TA100 and TA1537	33 to 10 000 µg/plate	Positive [(b)]	Mortelmans et al., 1986	Valid.
	Reverse mutation	*S. typhimurium* TA97 and TA102	0.1, 0.5, 1, 5, or 10 mg/plate (100, 500, 1 000, 5 000, or 10 000 µg/plate)	Weak Positive [(a)]	Fujita et al., 1992	Result is considered equivocal. Limited validity (the use of only two strains is not according to OECD guideline).
	DNA damage (SOS Chromotest)	*Escherichia coli* PQ37	5 mM (631 µg/ml)	Negative	Ohshima et al., 1989	The test system used is considered inappropriate, due to insufficient validity.

Chemical name [FL-no]	Test system	Test object	Concentration	Reported result	Reference	Comments [(d)]
	Sister chromatid exchange	Chinese hamster ovary cells	Up to 1.5 μmol/ml (12.6 to 189 μg/ml)	Positive [(c)]	Gava et al., 1989	Validity cannot be evaluated (insufficiently reported: number of cells analysed not reported. Statistical test used not reported). SCEs were reported as SCE per chromosome. Effect was less than twofold compared to control.
	Sister chromatid exchange	Human lymphocytes	Up to 1.0 mM (126.11 μg/ml)	Positive	Jansson et al., 1986	Validity cannot be evaluated. Relevance of test system for the evaluation of genotoxicity uncertain.
Ethyl maltol [07.047]	Reverse mutation	*S. typhimurium* TA 1535, TA1537, TA1538, TA98 and TA100	5 concentrations up to cytotoxicity, or max. 3 600 μg/plate	Negative [(a)]	Wild et al., 1983	Limited validity (result not reported in details, no TA102 or E. Coli).
	Reverse mutation	*S. typhimurium* TA98 and TA100	Up to 2 mg/plate (2 000 μg/plate)	Positive [(a)]	Bjeldanes and Chew, 1979	Valid.

(a): With and without metabolic activation
(b): With metabolic activation
(c): Without metabolic activation
(d): Validity of genotoxicity studies:
- Valid
- Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation)
- Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
- Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

4.5. Genotoxicity data (*in vivo*) considered by the Panel in FGE.213

Table 8: Genotoxicity (*in vivo*)

Chemical name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments [(a)]
Maltol [07.014]	Micronucleus formation	ddY Mouse bone marrow cells	Intraperitoneal	125, 250, or 500 mg/kg	Positive	Hayashi et al., 1988	Valid. The induction of micronuclei was up to about 10-fold compared with control
	Sex-linked Recessive Lethal Mutation	*Drosophila melanogaster*	Feed	6 000 ppm (6000 µg/ml)	Equivocal	Zimmering et al., 1989	Limited validity (only one exposure level tested). Test system considered of limited relevance.
	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	Feed	10 000 ppm (10 000 µg/ml)	Negative	Mason et al., 1992	Valid, however, test system considered of limited relevance.
	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	Injection	0.2 – 0.3 µl, 10 000 ppm (10 000 µg/ml)	Negative	Mason et al., 1992	Valid, however, test system considered of limited relevance.
Ethyl maltol [07.047]	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	420, 700, or 980 mg/kg	Negative	Wild et al., 1983	Limited validity (injected twice; only analysis at one time point; no PCE/NCE ratio reported).
	Micronucleus formation	NMRI mouse bone marrow cells	Intraperitoneal	980 mg/kg	Negative	Wild et al., 1983	Limited validity (single injection, analysis at three time points, no PCE/NCE ratio reported).
	Sex-linked recessive lethal mutation (Basc test)	*Drosophila melanogaster*	Feed	14, 25 or 50 mM	Negative	Wild et al., 1983	Limited validity (limited reporting, test system considered of limited relevance).

(a): Validity of genotoxicity studies:
- Valid
- Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation)
- Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
- Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too few experimental details provided)

4.6. Carcinogenicity studies considered by the Panel in FGE.213

Table 9: Carcinogenicity studies

Chemical name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments [(a)]
Ethyl maltol [07.047]	Rats; Male, Female 25/sex/group	Diet	0, 50, 100 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	Gralla et al., 1969	Valid. The study was performed before the introduction of OECD guidelines but is, however, considered valid. The NOAEL was 200 mg/kg bw/day, the highest dose tested
3-Ethylcyclopentan-1,2-dione [07.057]	Rats; Male, Female 50/sex/group	Diet	0, 30, 80 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	King et al., 1979	Valid. The study was performed before the introduction of OECD guidelines but is, however, considered valid. The NOAEL was 200 mg/kg bw/day, the highest dose tested

(a): Validity of genotoxicity studies:

- Valid
- Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation)
- Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
- Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too few experimental details provided)

4.7. Genotoxicity data (*in vitro*) considered by the Panel in FGE.213Rev1 and FGE.213Rev2

Table 10: Summary of additional *in vitro* genotoxicity data for FGE.213Rev1

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
β-Ionone [07.008]	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32–5000 µg/plate (a, b)	Negative	Ballantyne, 2011	Evidence of toxicity was observed in all strains at concentrations of 1000 µg/plate and greater in the absence and in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	10.24–1000 µg/plate (b, d) or (c, e)	Negative		Evidence of toxicity was observed in all strains at 1 000 µg/plate in the absence and presence of S9-mix, and in most cases these toxic effects also extended down to concentrations of 160 or 400 µg/plate. Study design complied with current recommendations.
	Micronucleus assay	Human peripheral blood lymphocytes	30–60 µg/ml (d, f) 80–120 µg/ml (e, f) 5–17.5 µg/ml (d, g)	Negative	Stone, 2011a	The top concentrations induced 50–60 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
Maltol [07.014]	Reverse mutation	*S. typhimurium* TA98, TA100 and TA102, TA1535 and TA1537	0.32–5000 µg/plate [a, b]	Negative	Ballantyne, 2012	Evidence of toxicity was observed in TA102 at concentrations of 1000 and 5000 µg/plate in the absence of S9-mix and at concentrations of 200 µg/plate and greater in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA1535 and TA1537	51.2–5000 µg/plate (b, d) or (c, e)	Negative		Toxicity was observed at 5000 µg/plate in strain TA100 only in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA102	20.48–5000 µg/plate (b, d) or (c, e)	Negative		Evidence of toxicity was observed at 5 000 µg/plate in the absence and presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus assay	Human peripheral blood lymphocytes	400–1262 µg/ml [d, f] 400–1262 µg/ml [e, f] 125–300 µg/ml [d, g]	Equivocal Positive Negative	Whitwell, 2012	The top concentrations in the 3 + 21 hours treatments in the absence and presence of S9-mix induced, respectively, 24 % and 19 % of toxicity. The top concentration in the 24 + 0 hours treatment in the absence of S9-mix induced 57 % toxicity. There was evidence of micronuclei induction when tested for 3 + 21 hours in the presence of S9-mix, while in absence of S9-mix the data were considered equivocal. However, no induction of micronuclei was observed in the continuous exposure test. Study design complies with OECD Guideline 487.
	Chromosomal aberration test	CHL cells	25, 50, 75 µg/ml (d, g) or (d, h)	Positive	Ishidate, 1988	Structural chromosomal aberrations were observed at the 2 highest concentrations tested.

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
β-Damascone [07.083]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.32–5000 µg/plate (a, b)	Negative	Bowen, 2011b	Toxicity was observed at 1 000 and/or 5 000 µg/plate across all strains in the absence and presence of S9-mix; no clear evidence of toxicity in TA100 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix.
		S. typhimurium, TA1535, TA1537 and TA102 *S. typhimurium* TA98, TA100	78.13–2500 µg/plate (b, d) or (c, e) 156.3–5000 µg/plate (b, d) or (c, e)	Negative Negative		Evidence of toxicity was observed at the highest three or four concentrations across all strains in the absence and presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix
		S. typhimurium TA98	19.3–1250 µg/plate (c, e)	Negative		Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, in the presence of S9-mix.
	Micronucleus assay	Human peripheral blood lymphocytes	8–22 µg/ml (d, f) 12–18 µg/ml (e, f) 6–9 µg/ml (d, g)	Equivocal(d, f) Positive (e, f) Equivocal(d, g)	Stone, 2012	Positive result was obtained in the 3 + 21 hour treatment in the presence of S9-mix. Study design complies with OECD Guideline 487.
Nootkatone [07.089]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.5–50 µg/plate (b, d) 1.5.5–150 µg/plate (b, e) 0.5–50 µg/plate (b, d) 0.5–150 µg/plate (c, e)	Negative	Marzin, 1998	Evidence of toxicity was observed at 50 µg/plate in all strains in the absence of S9-mix and at 150 µg/plate in all strains in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus assay	Human peripheral blood lymphocytes	50–80 µg/ml (d, f) 160–185 µg/ml (e, f) 10–24 µg/ml (d, g)	Negative	Stone, 2011b	The top concentrations in all parts of the study induced >50 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
2,6,6-Trimethylcyclohex-2-en-1,4-dione [07.109]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.32–5000 µg/plate [a, b]	Negative	Bowen, 2011a	Evidence of toxicity was observed at 1000 and/or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	156.3–5000 µg/plate [b, d] or [c, e]	Negative		Evidence of toxicity was observed in TA102 at 2500 and 5000 µg/plate. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	500–1522 µg/ml [d, f] 1000–1522 µg/ml [e, f] 300–550 µg/ml [d, g]	Negative	Lloyd, 2011	The top concentrations in the 3 + 21 hours in the absence and presence of S9-mix were 10 mM. The top concentration in the 24 + 0 hours in the absence of S9-mix induced 57 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

(a): With and without S9-mix metabolic activation
(b): Plate incorporation method
(c): Without S9-mix metabolic activation
(d): Pre-incubation method
(e): With S9-mix metabolic activation
(f): 3-hour incubation with a 21-hour recovery period
(g): 24-hour incubation with no recovery period
(h): 48-hour incubation with no recovery period

4.8. Genotoxicity data (*in vivo*) considered by the Panel in FGE.213Rev1 and FGE.213Rev2

Table 11: Summary of additional *in vivo* genotoxicity data submitted for FGE.213Rev1 and FGE.213Rev2

Chemical name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments
Maltol [07.014]	Micronucleus assay	Han Wistar Rat; M	Gavage	70, 350, 700 mg/kg bw/day [(a)]	Negative	Beevers, 2013a	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. However, evidence of target tissue exposure was inconclusive. The study was performed in compliance with OECD Guideline 474. A further plasma analysis was performed (Beevers, 2015) showing the systemic exposure of animals to maltol. Based on the new bioanalysis, results of the micronucleus assay were considered as negative.
	Comet assay	Han Wistar rat; M	Gavage		Negative		Mean percentage tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the comet and IWGT workshop, Japanese Centre for the Validation of Alternative Methods (JaCVAM) and current literature.
β-Damascone [07.083]	Micronucleus assay	Han Wistar rat; M	Gavage	125, 250 and 500 mg/kg bw/day [(a)]	Negative	Beevers, 2013b,c	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. The study was performed in compliance with OECD Guideline 474.
	Comet assay	Han Wistar rat; M	Gavage		Negative		Mean% tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the comet and IWGT workshop, Japanese Centre for the Validation of Alternative Methods (JaCVAM) and current literature.

(a): Administered via gavage in 3 doses at times 0, 24 and 45 hours with sacrifice and harvest at 48 hours

Documentation provided to EFSA

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Abbreviations

bw	Body Weight
CAS	Chemical Abstracts Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese Hamster Ovary (cells)
CHL	Chinese Hamster Lung (cells)
CoE	Council of Europe
EC	European Commission
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FISH	Fluorescence In Situ Hybridisation
FLAVIS (FL)	Flavour Information System (database)
GC–MSD	gas chromatography–mass selective detection
GLP	Good Laboratory Practice
ID	Identity
IOFI	International Organization of the Flavor Industry
IR	Infrared Spectroscopy
IWGT	International Workshops on Genotoxicity Testing
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei
MNBN	MicroNucleated BiNucleate cells
MNPCE	Micronucleated Polychromatic Erythrocytes
MS	Mass Spectrometry
MSDI	Maximised Survey-derived Daily Intake
MTD	Maximum Tolerated Dose
NCE	NormoChromatic Erythrocytes
NMR	Nuclear Magnetic Resonance
No	Number
NOEL	No Observed Effect Level
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
PHA	Phytohaemagglutinin
(Q)SAR	(Quantitative) Structure Activity Relationship
SCE	Sister Chromatid Exchange
WHO	World Health Organization